

**Amendments to the Claims:**

This listing of claims will replace all prior versions, and listings, of claims in the application:

**Listing of Claims:**

1-34. (cancelled)

35. (currently amended) A modified pneumolysin polypeptide comprising one or more amino acid substitutions, wherein the modification of the in a pneumolysin polypeptide comprises substituting at least one amino acid of having a sequence of SEQ ID NO:3, and wherein said substitution is wherein single amino acid substitutions occur at a position selected from the group consisting of positions 61, 148, and 195, or wherein substitutions of multiple more than one amino acids occur at positions selected from the group consisting of 17, 18, 33, 41, 45, 46, 61, 63, 66, 83, 101, 102, 128, 148, 189, 195, 239, 243, 255, and 257, and wherein when said modified pneumolysin polypeptide possesses only one substitution, said substitution is selected from the group consisting of positions 61, 148, and 195, and wherein said modified pneumolysin polypeptide having at least one amino acid substitution is soluble, elicits antibodies which are cross-reactive with wild-type pneumolysin, and has attenuated hemolytic activity.

36. (previously presented) The polypeptide according to claim 35, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.

37. (previously presented) The polypeptide according to claim 36, wherein the hemolytic activity is less than 1% compared to wild-type pneumolysin.

38-41. (cancelled)

42. (previously presented) The polypeptide according to claim 35, wherein the polypeptide is selected from the group consisting of pNVJ1, pNVJ20, pNVJ22, pNVJ45, pNVJ56, pNV103, pNV207, pNV111, and pNV211.

43. (previously presented) Modified pneumolysin polypeptide pNVJ1.

44. (previously presented) Modified pneumolysin polypeptide pNVJ20.

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We claim:

1. A modified pneumolysin polypeptide having attenuated hemolytic activity wherein said modified pneumolysin polypeptide is obtained by:

- a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid molecules in host cells;
- b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
- c) identifying the modified pneumolysin polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.

2. A modified properly-refolded pneumolysin polypeptide having attenuated hemolytic activity comprising an amino acid sequence of type 14 pneumolysin wherein at least one amino acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in attenuation of the hemolytic activity of the modified pneumolysin polypeptide.

3. The modified pneumolysin polypeptide of claim 2, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.

4. A modified pneumolysin polypeptide according to claim 3, comprising at least one amino acid substitution in the amino acid sequence of Formula I, <sup>SEQ ID NO. 3</sup> at residue positions 61, 148, or 195 or the combination of substitutions at residue positions 33, 46, 83, 239 and 257, said Formula I comprising

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1 Asp Lys Val  
5  no period.

- 35 5. The modified pneumolysin according to claim 4,  
wherein a single amino acid substitution is made and  
the substituted amino acid is selected from the group  
consisting of proline or hydroxyproline for position  
61; lysine, arginine or histidine for position 148 and  
40 leucine, glycine, alanine, isoleucine or valine for  
position 195.
6. The modified pneumolysin according to claim 3,  
wherein the substituted amino acids are selected from



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T-134→C, A-305→G, A-566→G and T-583→G;

T-583→G;

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T-583→A;

T-443→A;

and

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T-181→C.

18. The recombinant nucleic acid molecule of claim 16 as contained in a vector such as a plasmid, cosmid, bacteriophage or yeast artificial chromosome.

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19. A microorganism comprising the nucleic acid molecule of claim 16.

20. The microorganism according to claim 19, wherein the microorganism is selected from the group consisting of bacteria, yeast, mammalian or insect cells.

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21. The microorganism according to claim 20, wherein the microorganism is *E. coli*.

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22. The modified pneumolysin polypeptide of <sup>claim 2</sup>~~claim 1~~, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.

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23. The modified pneumolysin conjugate of claim 22, wherein the polysaccharide is from a bacteria selected from the group consisting of a Haemophilus influenzae type b; meningococcal group A, B or C; group B streptococcus types Ia, Ib, II, III, V or

VIII and pneumococcal.

24. A vaccine comprising at least one pneumolysin polypeptide of claim 1 and a pharmaceutically acceptable carrier.

5 25. The vaccine according to claim 24, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.

10 26. The vaccine according to claim 25, wherein the polysaccharide is derived from a bacteria selected from the group consisting of Haemophilus influenzae type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus serotypes Ia, Ib, II, III, V, or VIII; or one or more of serotypes 1-23 of *S. pneumoniae*.

15 27. A method for killing bacteria comprising contacting said bacteria with antibodies to an immunogenic molecule comprising the modified pneumolysin according to claim 1 in the presence of complement.

20 28. The method according to claim 27, wherein the immunogenic molecule is a polysaccharide-polypeptide conjugate wherein the polysaccharide is a bacterial capsular polysaccharide.

25 29. A method for immunization of mammals comprising administering the vaccine of claim 24 to said mammals.

30. A method for obtaining modified pneumolysin polypeptides having reduced hemolytic activity and